Changes in diversity, composition and assembly processes of soil microbial communities during *Robinia pseudoacacia* L. restoration on the Loess Plateau, China

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Abstract: *Robinia pseudoacacia* L. (RP) restoration has increased vegetation cover in semi-arid regions on the Loess Plateau of China, but ecological problems have also occurred due to RP restoration, such as reduced soil moisture. Further, it is still uncertain how microbial diversity, composition and assembly processes change with RP restoration in semi-arid regions. Therefore, amplicon sequencing of small subunit ribosomal ribonucleic acid (16S rRNA) and internal transcribed spacer (ITS) genes was performed to study soil bacterial and fungal diversity, composition and assembly processes at four study sites with different stand ages of RP plantations (Y10, RP plantation with stand ages less than 10 a; Y15, RP plantation with stand ages approximately 15 a; Y25, RP plantation with stand ages approximately 25 a; and Y40, RP plantation with stand ages approximately 40 a) along a 40-a chronosequence on the Loess Plateau. The diversity of soil bacteria and fungi increased significantly during the restoration period from 10 to 15 a (P<0.05). However, compared with Y15, bacterial diversity was lower at Y25 and Y40, and fungal diversity remained stable during the restoration period between 25 and 40 a. The relative abundances of Proteobacteria and Ascomycota increased during the restoration period from 10 to 15 a. Conversely, after 15 a of restoration, they both decreased, whereas the relative abundances of Actinomycetes, Acidobacteria and Basidiomycota gradually increased. The variations in soil bacterial communities were mainly related to changes in soil total nitrogen, nitrate nitrogen and moisture contents, while soil fungal communities were mainly shaped by soil organic carbon and nitrate nitrogen contents. Bacterial communities were structured by the heterogeneous selection and stochastic process, while fungal communities were structured primarily by the stochastic process. The RP restoration induced an increase in the relative importance of heterogeneous selection on bacterial communities. Overall, this study reveals the changes in microbial diversity, community composition and assembly processes with RP restoration on the Loess Plateau and provides a new perspective on the effects of vegetation restoration on soil microbial communities in semi-arid regions.

Keywords: *Robinia pseudoacacia*; microbial diversity; community structure; community assembly; restoration; semi-arid regions

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1 Introduction

Vegetation restoration of degraded farmlands has been widely implemented to improve soil quality and enhance the ecological and economic values of land in arid and semi-arid regions with fragile ecosystems (Knoke et al., 2014; Rong et al., 2020). *Robinia pseudoacacia* L. (RP), a nitrogen (N)-fixing tree species, has been selected as an important pioneer species for the conversion of farmland to forests. RP is planted worldwide due to its tolerance to diverse soil conditions, surviving in soil with pH ranging from extremely acidic to strongly alkaline, as well as in soil with base-saturation levels ranging from medium to high (Rédei et al., 2014; Vitkova et al., 2015). During RP restoration, originally barren land undergoes a series of soil processes, including carbon (C) and N input and transformation, and nutrient accumulation (Jin et al., 2016; Papaioannou et al., 2016). Soil microbes play important roles in mediating these processes. For example, soil fungi complete the decomposition of plant litter, and bacteria primarily participate in the biogeochemical cycle of soil C and N (Romaní et al., 2006; de Menezes et al., 2017). In addition, metrics related to microbial communities (i.e., diversity, microbial biomass and presence of beneficial microbes) can be used as important indicators to evaluate the success of the restoration of degraded ecosystems. For example, soil bacterial diversity can be recovered to pre-farmland levels after approximately 20 a of secondary succession on abandoned farmland and this process is highly dependent on the availability of soil nutrients (Zhang et al., 2016). Soil microbial biomass, one of the main nutrient sources of plants in nutrient-restricted areas, accumulates during restoration, and it is thus considered as an important indicator of soil fertility (Singh et al., 2009; Shao et al., 2019). Increases in the diversity of N-fixing microbes associated with RP restoration were significantly positively correlated with the amount of available N in soils on the Loess Plateau, China (Xu et al., 2019). Therefore, studying soil microbial communities can increase our understanding of the succession of ecosystems and enable restoration.

RP restoration significantly affects the diversity and composition of soil microbial communities (Xu et al., 2019; Xu et al., 2020a). On the one hand, during RP restoration, the increase in plant litter provides more substrates for decomposition by microbes (Xu et al., 2020b; Dong et al., 2021). On the other hand, RP restoration changes soil properties, such as reducing the pH and bulk density of soil, as well as increasing the C and N contents, which have significant impacts on the metabolism and activity of soil microbes. For example, Liu et al. (2018) reported that during a 35-a RP restoration, Acidobacteria eventually replaced other species to become the dominant phylum in soil, and phosphorus (P) content was the main factor shaping the composition of microbial communities. Xu et al. (2019) found that compared with farmland, 42 a of RP restoration significantly improved the diversity index and species richness of soil bacteria and fungi. Recently, however, the problem of soil moisture (SM) decline following RP restoration has become particularly serious. Several studies have found that SM showed a linear decreasing trend with increasing recovery age in different precipitation zones (400.0–600.0 mm), which led to increased RP plantation mortality and land degradation (Liang et al., 2018; Su et al., 2019; Wen and Théau, 2020). These findings suggest that the response of soil microbes to vegetation restoration may be more complex in semi-arid regions. Therefore, it is necessary to reassess the response of soil microbial communities to RP restoration in semi-arid regions.

Microbial community assembly is an important research topic in ecology (Zhou and Ning, 2017). In contrast to the body of knowledge regarding large organisms, understanding on the mechanisms of microbial community assembly is still very limited. Currently, it is generally believed that microbial community assembly is driven by both deterministic process (environmental selection) and stochastic process (dispersal, ecological drift and diversification). Moreover, the importance of these two types of processes has been quantified by using many models (Dumbrell et al., 2010; Stegen et al., 2013). Liu et al. (2021) found that the assembly of prokaryotic microbial communities was controlled by deterministic process in the early succession of subtropical forests. In contrast, the relative influence of stochastic process increased

in the later stage. Osburn et al. (2021) revealed that the assembly of soil bacterial and fungal communities was primarily affected by deterministic process in historically disturbed forests (e.g., logging and agricultural conversion). In pristine forests that have remained undisturbed for approximately 100 a, stochastic process is more important. Despite preliminary studies of soil microbial assembly processes in forests, the assembly processes along a chronosequence (i.e., RP restoration) are still unclear, especially in semi-arid regions. This is crucial for understanding the drivers of soil microbial community succession.

The Loess Plateau is a semi-arid region in China with severe soil erosion and ecological fragility (Zheng and Wang, 2014). RP has been widely planted in this region and has been a pioneer species for afforestation of farmland since the 1970s (Zhang et al., 2019). Although RP restoration increased the coverage of local vegetation, it also caused a series of environmental problems, e.g., declines in SM (Zhao et al., 2018). To date, the variation in microbial communities and their ecological processes during RP restoration on the Loess Plateau have not been fully explored. Here, based on the spatial (as opposed to temporal) method, we studied soil bacterial and fungal communities at RP plantations with different stages on the Loess Plateau. Our aims were to (1) determine the variation in the diversity and composition of soil bacterial and fungal communities at RP plantations with different stages; (2) illustrate important soil parameters related to this variation; and (3) determine the ecological process that drives the assembly of soil bacterial and fungal communities.

2 Materials and methods

2.1 Study area

The study area (109°28′12″–110°22′02″E, 36°27′08″–38°47′36″N) is located in a loess hilly and gully region on the Loess Plateau, China. The loess thickness in this area is 30–180 m and the soil type is mainly loessial soil developed from loess parent material. The area is characterized by a temperate and semi-arid climate, with the annual mean temperature of 8.8 ℃ and the mean annual precipitation of 505.3 mm. The average altitude in the study area is 1371.6 m, the average annual sunshine duration is 2395.6 h, and the annual frost-free period is 157 d.

2.2 Soil sampling

In April 2019, we identified four RP plantations at four study sites along a chronosequence as follows: Y10, RP plantation with stand ages less than 10 a; Y15, RP plantation with stand ages approximately 15 a; Y25, RP plantation with stand ages approximately 25 a; and Y40, RP plantation with stand ages approximately 40 a. Apart from differences in stand ages, the four plantations were similar in terms of geographical characteristics and soil types, and they were all agricultural lands with maize and wheat as the main crops before RP restoration. The different plantations are located more than 5 km apart. After RP restoration, these plantations were rarely managed by humans; thus, their development can be considered a natural restoration process. Previous study has shown that on the Loess Plateau, the mature period of RP plantations is 20 a (Wei et al., 2018). Therefore, Y25 and Y40 represent the later restoration stage. More information on the study sites is shown in Table S1.

To avoid the problem of ecological pseudoreplication as much as possible, we established five 10 m×10 m plots with distance no more than 50 m from each other at each site, and the sampling area at each site was approximately 100 m×100 m. In each plot, five locations for soil cores were randomly selected. First, the litter on the surface of soil was carefully removed. Then, bulk soil samples were collected from the top soil with depth of 0–20 cm using a 5 cm diameter stainless steel corer. Finally, the litter and roots in each soil sample were removed, and the five individual soil samples collected from the same plot were sieved using a 2 mm mesh and fully mixed into a single soil sample. In this way, a total of 20 soil samples were obtained. Each soil sample was divided into two subsamples. The first subsample was transported to the laboratory on ice and stored at –80 ℃ until deoxyribonucleic acid (DNA) extraction was performed. The second subsample was used for soil physical and chemical analyses.

2.3 Analyses of soil physical and chemical properties

Soil pH was measured (1.0:2.5 soil/water) using a pH metre (IS126, Insmark Inc., Shanghai, China). SM was measured by a FieldScout time domain reflectometry (TDR) moisture metre sensor (TDR 300, Spectrum Technologies Inc., Plainfield, USA). Soil organic carbon (SOC) was determined by the $K_2Cr_2O_7$-H_2SO_4 oxidation method (Nelson and Sommers, 1982). Total nitrogen (TN) was determined using the Kjeldahl method (Bremner and Mulvaney, 1982). Nitrate nitrogen (NO_3^--N) and ammonium nitrogen (NH_4^+-N) were extracted with 1 mol/L KCl and determined by a continuous flow analytical system (AA3, SEAL Analytical Ltd., Norderstedt, Germany). Available phosphorus (AP) was determined by the molybdenum antimony colorimetric method after extraction with 0.5 mol/L $NaHCO_3$ (Olsen and Sommers, 1982).

2.4 DNA extraction and sequencing

We extracted soil microbial DNA from the 20 soil samples (approximately 0.5 g for each) using the E.Z.N.A.® soil DNA Kit (D5625, Omega Bio-Tek Inc., Norcross, USA) according to the manufacturer's protocols. The final DNA concentration and purification were determined by a Nano Drop 2000 UV-Vis spectrophotometer (Thermo Scientific Inc., Massachusetts, USA), and DNA quality was assessed by 1% agarose gel electrophoresis. All DNA samples were stored at –80 ℃ until the following analysis. Bacterial 16S rRNA (ribosomal ribonucleic acid) (V4–V5 regions) and fungal ITS (internal transcribed spacer) RNA (ribonucleic acid) genes were amplified with primers 515F/907R (Biddle et al., 2008) and ITS1F/ITS2R (Adams et al., 2013) with barcodes, respectively. Polymerase chain reaction (PCR) was performed by a PCR instrument (GeneAmp 9700, ABI, Carlsbad, USA) in triplicate to obtain as many target genes as possible for sequencing. Additional details of the PCR system and conditions were as previously reported (Zhou et al., 2019). The PCR products from three technical replicates were fully mixed and purified by an Agarose Gel DNA Extraction Kit v4.0 (Takara Bio Inc., Tokyo, Japan). Finally, all purified PCR products (20 samples) were mixed at equimolar concentrations and sequenced on an Illumina HiSeq 2500 platform (Illumina Inc., San Diego, USA). Amplicon sequencing was performed by the Major Biological Institute (Shanghai, China).

2.5 Bioinformatic analysis

Raw sequence files were quality-filtered by Trimmomatic and merged using Fast Length Adjustment of Short Reads (FLASH) in terms of the following criteria: operational taxonomic units (OTUs) were clustered with a 97% similarity cut-off using Usearch v7.0, and single sequences and chimaeras were removed during the clustering process. According to the Silva 128 reference database, we used the Ribosomal Database Project (RDP) classifier for annotation of each OTU's representative sequence. The complete sequences have been submitted to the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI), with accession numbers PRJNA705738 (16S rRNA) and PRJNA705794 (ITS). To analyze α diversity, we rarefied the sequence number of each sample at the same value using the function "multiple_rarefactions.py" to eliminate the difference caused by sequencing depth. Shannon and Chao indices were calculated to characterize α diversity using the function "alpha_diversity.py". We further calculated Bray-Curtis distance using the function "beta_diversity.py" to quantitate the variation in microbial communities based on the rarefied OTU table. The above functions were performed with the QIIME v1.9.1 pipeline.

2.6 Calculation of ecological processes

To assess the relative importance of stochastic and deterministic processes, we investigated community phylogenetic turnover that is defined as the phylogenetic distance between OTUs in microbial communities (β-mean nearest taxon distance (βMNTD)). An expected βMNTD (null-βMNTD) was generated using the "null-model" (based on 9999 iterations), and then the observed βMNTDs (obs-βMNTDs) between microbial communities were calculated. Beta nearest taxon index (βNTI) was calculated to quantify the magnitude of the deviation between

obs-βMNTD and null-βMNTD, as well as the sign (positive or negative). The value of βNTI larger than 2 indicates that heterogeneous selection strongly drives the microbial community assembly, and the value of βNTI less than –2 indicates that homogeneous selection strongly drives the microbial community assembly. Thus, an absolute value of βNTI larger than 2 (|βNIT|>2) implies that the deterministic process strongly drives the microbial community assembly. The microbial community assembly is considered to result from stochastic process if |βNTI|<2. The detailed calculation methods of null-βMNTD, obs-βMNTD and βNTI can be found in Dini-Andreote et al. (2015).

2.7 Statistical analyses

Significant differences (P<0.05) in soil properties and α diversity indices between RP plantations with different stand ages were tested by one-way analysis of variance (ANOVA). The data were log transformed if necessary. Pearson correlation analysis was used to summarize the relationship between microbial diversity indices and soil properties. Principal component analysis (PCA) was used to describe the differences between soil properties among different RP plantations. Principal coordinate analysis (PCoA) based on the Bray-Curtis distance was conducted to visualize the variations in community structure, while the Adonis test was used to examine for significant differences in community structure between RP plantations with different stand ages (based on 999 iterations). Redundancy analysis (RDA) was conducted to evaluate the effects of soil properties on bacterial and fungal community structures using CANOCO software v5. Linear regression was further used to examine the relationship between the temporal distance among samples and similarity in microbial composition (Bray-Curtis distance). A forward selection procedure was performed to reveal the significant explanatory variables by Monte Carlo permutations. All the statistical analyses mentioned above were performed in R 3.6.3 software without further modification.

3 Results

3.1 Soil properties

The contents of SOC, TN, NH_4^+-N and AP increased gradually from Y10 to Y40, with significant increases of 1.60-, 1.50-, 1.00- and 0.92-fold, respectively (P<0.05; Table 1). The content of NO_3^--N decreased gradually from Y10 to Y40 (P<0.05). The soil pH continuously decreased from Y10 to Y40. SM was the lowest at Y25 and Y40 and the highest at Y15 (P<0.05). Based on the measured soil characteristics, the four stand ages of RP plantations were distinctly separated along the first principal component (PC) coordinate axis (Fig. S1), explaining more than 78.0% of the total variation. Overall, these results indicated that the restoration of RP significantly altered soil properties.

Table 1 Soil properties of *Robinia pseudoacacia* (RP) plantations with different stand ages

Soil property	RP plantation			
	Y10	Y15	Y25	Y40
pH	8.76±0.05^{a}	8.59±0.13^{b}	8.53±0.09^{bc}	8.48±0.12^{c}
SM (%)	10.79±0.55^{b}	14.20±0.99^{a}	9.65±0.34^{c}	9.57±0.46^{c}
SOC (mg/g)	5.94±0.42^{d}	6.89±0.21^{c}	13.86±0.31^{b}	15.89±0.67^{a}
TN (mg/g)	0.66±0.05^{d}	0.76±0.03^{c}	1.49±0.04^{b}	1.66±0.06^{a}
NO_3^--N (μg/g)	1.95±0.15^{a}	1.79±0.31^{a}	1.12±0.34^{b}	1.60±0.77^{ab}
NH_4^+-N (μg/g)	0.46±0.04^{c}	0.40±0.05^{c}	0.71±0.15^{b}	0.92±0.09^{a}
AP (μg/g)	2.64±0.47^{c}	1.90±0.36^{c}	3.94±1.17^{b}	5.09±0.75^{a}

Note: Y10, Y15, Y25 and Y40 indicate RP plantations with stand ages of 10, 15, 25 and 40 a, respectively. SM, soil moisture; SOC, soil organic carbon; TN, total nitrogen; NO_3^--N, nitrate nitrogen; NH_4^+-N, ammonium nitrogen; AP, available phosphorous. Mean±SE; n=5. Different lowercase letters within the same row indicate significant differences of soil properties (P<0.05) among RP plantations with different stand ages based on one-way analysis of variance (ANOVA) followed by the least significance difference (LSD) test.

3.2 Diversity of microbial communities

After quality trimming and chimaera removal, the data set was found to contain a total of 794,499 high-quality bacterial sequences and 1,011,433 high-quality fungal sequences for the soil samples. The average numbers of bacterial OTUs at Y10, Y15, Y25 and Y40 were 2452 (±18), 2493 (±29), 2277 (±86) and 2471 (±183), respectively. The average numbers of fungal OTUs at Y10, Y15, Y25 and Y40 were 495 (±36), 614 (±32), 708 (±28) and 759 (±85), respectively. A Venn diagram showed that 3183, 2975 and 2988 bacterial OTUs at Y15, Y25 and Y40 were shared with Y10, respectively, and 639, 590 and 559 fungal OTUs at Y15, Y25 and Y40 were shared with Y10, respectively (Fig. S2), suggesting that compared with Y10, the similarity of microbial communities generally decreased with restoration time. The rarefaction curves appeared to flatten, and Good's coverage reached 99.98%, collectively indicating that the amount of sequencing data was reasonable.

For bacteria, the Shannon and Chao indices both reached their peak values at Y15 and then significantly decreased to their lowest values at Y25 and Y40 (P<0.05; Fig. 1a and b). In contrast, the Shannon and Chao index values for fungi showed a sharp increasing tendency from Y10 to Y25, with significant increases of 19.8% and 29.7% (P<0.05), respectively, and both of them exhibited a slightly increasing trend from Y25 to Y40 (P>0.05; Fig. 1c and d). Pearson correlation analysis (Table S2) revealed that SM was significantly positively related to the bacterial Shannon and Chao indices, while the increases in SOM, TN, NH_4^+-N and AP were significantly negatively related to the bacterial Shannon index (P<0.05). For fungi, the increases in SOM, TN, NH_4^+-N and AP were all significantly positively related to the Shannon and Chao indices, and the decrease in pH was significantly negatively related to the Shannon and Chao indices (P<0.05).

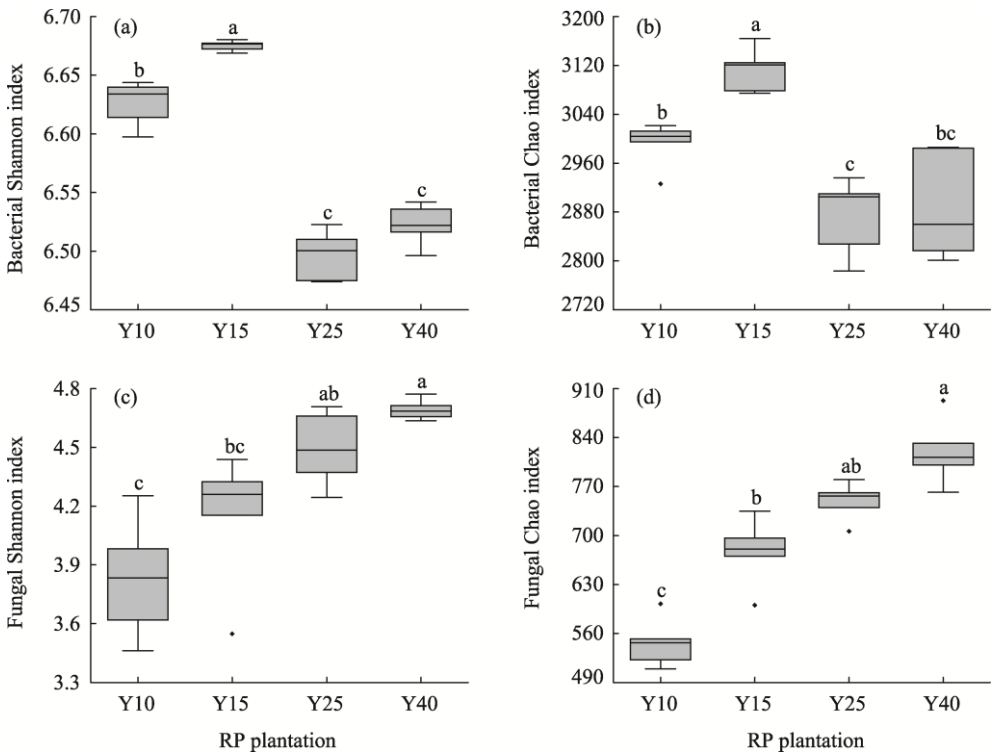

Fig. 1 Diversity indices of soil bacterial and fungal communities at four *Robinia pseudoacacia* (RP) plantations with different stand ages. (a), Shannon index of bacterial communities; (b) Chao index of bacterial communities; (c) Shannon index of fungal communities; (d) Chao index of fungal communities. Y10, Y15, Y25 and Y40 indicate RP plantations with stand ages of 10, 15, 25 and 40 a, respectively. Different lowercase letters indicate significant differences (P<0.05) among RP plantations with different stand ages. The box plot conveys the distribution of data values, including the median, the approximate quartiles, and the lowest and highest data (Williamson et al., 1989). The points in the box plot represent outliers.

3.3 Relative abundance of dominant phyla

At the bacterial phylum level (Fig. 2a), Proteobacteria (19.1%–34.5%), Actinobacteria (21.9%–32.8%) and Acidobacteria (14.3%–20.4%) were the dominant phyla across different RP plantations. The relative abundance of Proteobacteria was lowest at Y25 and Y40 and highest at Y15 (P<0.05). The relative abundance of Actinobacteria was highest at Y25 and Y40 and lowest at Y10 (P<0.05), and the relative abundance of Acidobacteria reached the highest value at Y40 (P<0.05). At the fungal phylum level (Fig. 2b), Ascomycota (66.2%–75.0%) was the dominant phylum across different RP plantations, followed by Zygomycota (15.0%–20.8%) and Basidiomycota (3.9%–12.3%). The relative abundance of Ascomycota significantly decreased from Y15 to Y25 and that of Basidiomycota increased gradually from Y10 to Y40 (P<0.05).

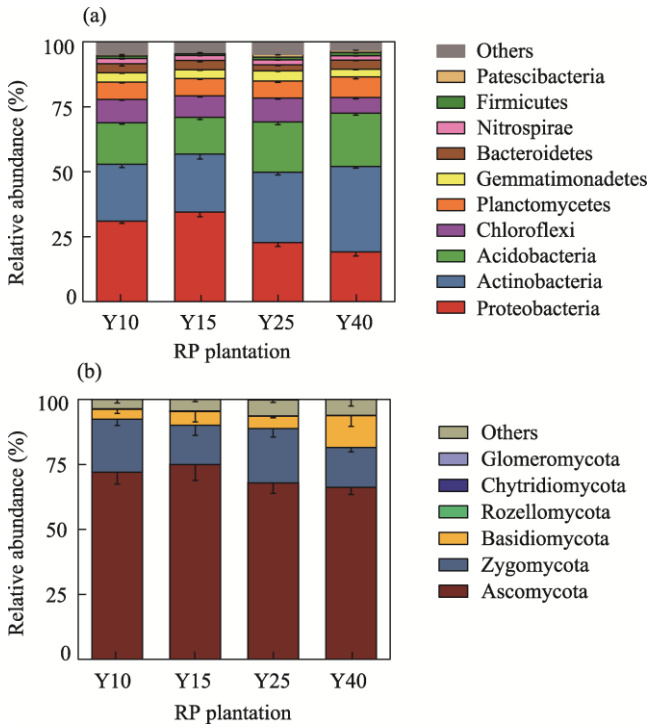

Fig. 2 Relative abundance of dominant bacteria (a) and fungi (b) at the phylum level at four RP plantations with different stand ages. The relative abundance level less than 1% and undefined phyla were defined as "Others". The error bars within the different colour blocks represent the standard variance of the species relative abundance (n=5).

3.4 Microbial community variation and determinants

Based on the Bray-Curtis distance (Fig. 3a and b), PCoA showed that bacterial and fungal community structures were clearly separated along the first and second axes among the four RP plantations. Accordingly, the Adonis test showed that bacterial and fungal community structures across years were extremely significantly different (P<0.05; Table S3). Linear regression analyses described the changes in microbial β diversity over time. The results revealed that bacteria showed a larger slope (temporal turnover) than fungi based on the Bray-Curtis distance (Fig. S3). According to RDA (Fig. 3c and d), the variations in bacterial and fungal community compositions were mostly affected by the variations in soil factors, which explained 67.1% and 56.7% of the bacterial and fungal community variance, respectively. According to forward selection (Table S4), the dissimilarities in TN, NO_3^--N and SM were closely related to the dissimilarity of bacterial community composition across the RP plantations (P<0.01), and those of SOC and NO_3^--N were closely related to the dissimilarity of fungal community composition across the RP plantations.

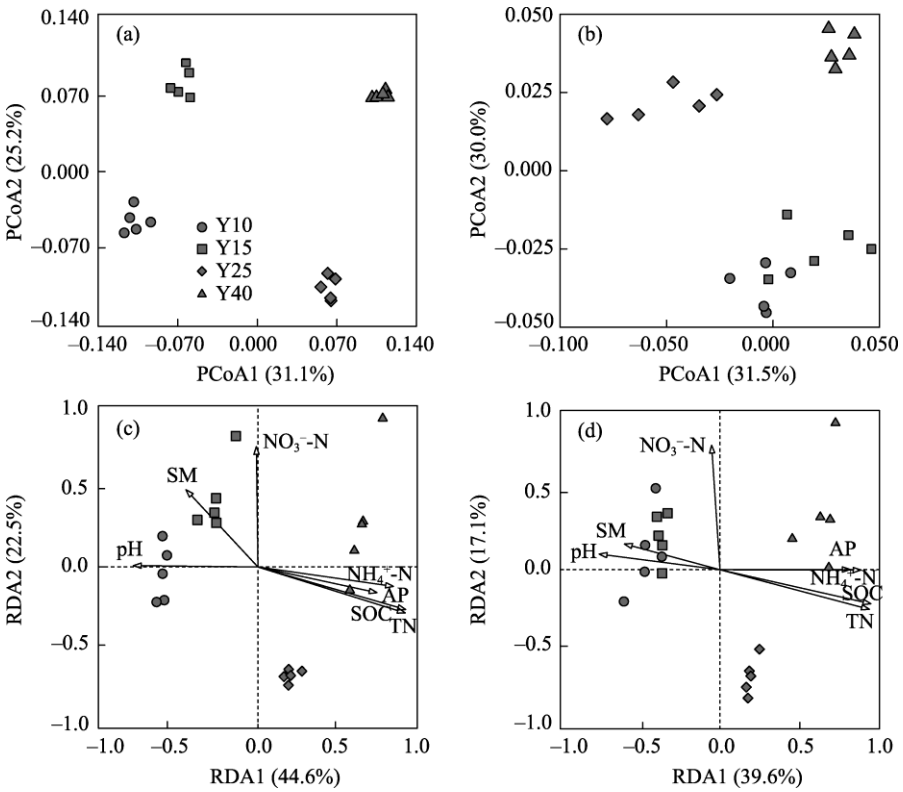

Fig. 3 Dissimilarities of soil microbial communities and the relationships between soil microbial communities and soil properties at four RP plantations with different stand ages. (a), principal coordinate analysis (PCoA) based on the bacterial community Bray-Curtis dissimilarities between samples; (b), PCoA based on the fungal community Bray-Curtis dissimilarities between samples; (c), redundancy analysis (RDA) between bacterial communities and soil properties; (d), RDA between bacterial communities and soil properties. SM, soil moisture; SOC, soil organic carbon; TN, total nitrogen; NO_3^--N, nitrate nitrogen; NH_4^+-N, ammonium nitrogen; AP, available phosphorous.

3.5 Assembly processes of microbial communities

We compared the relative importance of the two types of assembly processes (deterministic and stochastic processes) of bacterial and fungal communities at different RP plantations based on the βNTI. The assembly of bacterial communities was driven by both deterministic and stochastic processes (Fig. 4), and the deterministic process gradually became the main force (50.0% to 70.0% from Y10 to Y40) with restoration time. Surprisingly, all βNTI values of fungal communities were between –2 and 2 during the restoration time period from 10 to 25 a, indicating that the stochastic process dominated the assembly of fungal communities. The deterministic process became one of the forces (20.0%) for the assembly of fungal communities at Y40.

4 Discussion

During the early RP restoration period (stand ages from 10 to 15 a), the α diversity index of soil bacteria and fungi showed an upwards trend (Fig. 1). This is consistent with the results of previous studies (Liu et al., 2018; Xu et al., 2019; Xu et al., 2020b). Similarly, the restoration of other types of vegetation, such as *Platycladus orientalis* and *Masson pine*, also increases soil microbial α diversity (Sun et al., 2019; Hu et al., 2020). This increase might occur because vegetation restoration in the early period promotes more diverse ecological niches and high availability of resources for microbes (Zuo et al., 2016). However, in this study, we found that the variation patterns of soil microbial α diversity during the later RP restoration period (stand ages from 15 to 40 a) were not consistent with those during the early RP restoration period (Fig. 1).

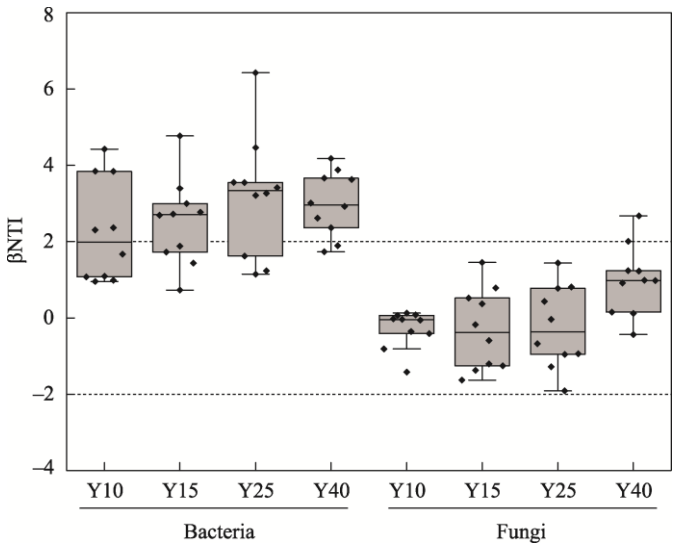

Fig. 4 Distribution of the microbial beta nearest taxon index (βNTI) at four RP plantations with different stand ages. The box plot conveys the distribution of βNTI values, including the median, the approximate quartiles, and the lowest and highest of values (Williamson et al., 1989). In addition, βNTI values for all samples (points in box plot) are shown. An absolute value of βNTI larger than 2 (|βNIT|>2) indicates that deterministic process strongly drives the microbial community assembly, while the microbial community assembly is considered to result from stochastic process if |βNTI|<2.

Liang et al. (2018) proposed that large-scale (an area of approximately 6.4×10^5 km^2) and long-term (more than 30 a) vegetation restoration leads to a decrease in SM on the Loess Plateau, and our study also found a decline in SM during the later restoration period (Table 1). SM was an important resource for the survival of microbes. The reduction in SM directly affected the metabolic activities of microbes and decreased the utilization of microbial C and N resources (Schimel, 2018). Moreover, we found that there was a significant positive correlation between bacterial diversity index and SM during the RP restoration period, while there were significant negative correlations of bacterial diversity index with SOC and soil nutrients (TN, NH_4^+-N and AP; Table S2). Compared to bacteria, soil fungi were more resistant to the declines in SM (de Vries et al., 2018), which may explain why the fungal α diversity only exhibited a slow trend during the RP restoration period (Fig. 1). Furthermore, Pearson correlation analysis showed that the fungal α diversity was significantly positively related to SOC and soil nutrients but negatively related to soil pH (Table S2), suggesting that the soil fungal α diversity indices were more dependent on SOC, soil nutrients and pH during the RP restoration period. Notably, the fungal α diversity indices increased slowly during the later restoration period (Fig. 1). Similar to the dynamics of the secondary plant succession, the increased lignin in litter indicated the decreased resource availability of soil fungi, which may limit the number of some fungi (Knops and Tilman, 2000; Cline and Zak, 2015). In addition, due to the increased dominance of a particular tree species, the narrowing of the range of organic substrates in soil during the late succession may limit the number of niches for saprophytic fungi (Zak et al., 2003). Thus, we speculate that variations in litter quality and vegetation composition might limit the fungal diversity despite improvements in soil nutrients and pH.

Results of RDA showed that soil N and SM were more closely related to the variations in bacterial community compositions (Fig. 3; Table S4). This is because soil nutrients and water are important resources for microbes. Changes in resource availability directly affect the metabolism and compositions of microbes (Tiemann et al., 2011; Crits-Christoph et al., 2013; Banerjee et al., 2020). These results have been extensively studied in terrestrial ecosystems. During the early RP restoration period, soil N showed an overall upwards trend. Sufficient labile substrates are conducive to the growth of Proteobacteria, a type of microbe that have faster access to substrates than other microbes (Fierer et al., 2007). Therefore, after 15 a of restoration, the reduction in soil

NO_3^--N might hinder the growth of eutrophic microbes. In addition, the decline in SM stimulated the growth of some drought-tolerant bacteria, such as Actinobacteria and Acidobacteria, which were considered as the dominant phyla in harsh and stressful environments (Barka et al., 2016; Arenas et al., 2021). For fungi, SOC content was closely related to the community compositions. This result is supported by the study of Liu et al. (2021), who found that during vegetation restoration, the types of SOC become more complex and are difficult to degrade. However, only a small number of fungi can secrete enzymes that catalyse the degradation of large organic C molecules, such as lignin. To date, the microbes that can secrete these enzymes mainly exist in the higher Basidiomycota (Purahong et al., 2016; Yang et al., 2017). Therefore, in this study, we found that during the later RP restoration period, the relative abundance of Ascomycota decreased, while the relative abundance of Basidiomycota began to increase. As was the case for fungi, soil NO_3^--N was also an important factor (Table S4). In terrestrial ecosystems, reduced available N content often leads to intense competition between microorganisms and plants (Wang et al., 2021). In this study, as the amount of available N increased, the classification and functional characteristics of soil fungi changed; for example, the relative abundance of mycorrhizal fungi was reduced (Leff et al., 2015).

Based on our calculated βNTI values, we found greater effects of the deterministic process on bacterial communities than on fungal communities. It has also been previously reported that bacteria are more susceptible to environmental factors than fungi (Powell et al., 2015; Wang et al., 2020). Many environmental factors can shape bacterial communities on spatial or temporal scales, such as climate (Guo et al., 2018), soil pH (Delgado-Baquerizo et al., 2018), soil nutrient availability (Leff et al., 2015), SM (Ma et al., 2015), etc. In this study, we found that SM was an important nonbiological factor that drove bacterial diversity and community compositions but did not have the same effect on fungi (Tables S2 and S4). Environmental factors may have less impact on fungal communities. This may be because fungi promote the acquisition of SM and soil nutrients through complex hyphal networks, which in return make them more resilient to environmental changes than bacteria (Yuste et al., 2011). Consistent with this conclusion, we found that during the 40-a restoration of RP plantations, the turnover rate of soil fungal communities was lower than that of soil bacterial communities (Fig. S3). In addition, some species of fungi can produce tens of billions of spores per square kilometer each year, promoting diffusion in ecosystems (Peay et al., 2012). In short, the differences in the community assembly of bacteria and fungi may reflect the differences in the life strategies (e.g., nutrient acquisition) of bacteria and fungi during the RP restoration period.

In addition, we found that the relative importance of the deterministic effect on bacterial communities gradually increased with the increasing restoration time. During the initial RP restoration period (5 a), the historical manual farmland management, such as ploughing, fertilization and irrigation, may provide an ideal condition for the diffusion of bacteria (Li et al., 2021). Previous study has suggested that the importance of the stochastic process generally increases as the supply of SOC increases (Chase, 2010). However, this study suggested that the relative importance of the deterministic process was negatively correlated with SOC content. This may be because RP restoration had a stronger selection effect on bacteria than the supply of SOC by reducing soil NO_3^--N and SM. Further work is needed to study the thresholds of these environmental factors that trigger a decrease in the importance of the stochastic process in bacterial communities. In addition, we observed that the heterogeneous selection effect of bacteria was strengthened during the RP restoration period (Fig. 4), which may be related to the topography of the study sites. Generally, there are large differences in SM and soil nutrients between the top and bottom of a slope, especially in the erosion-prone Loess Plateau region (Qiu et al., 2021). Therefore, the environmental heterogeneity in the region may gradually increase with the increasing restoration time.

5 Conclusions

The diversity of soil bacteria and fungi showed an increasing trend during the early RP restoration

period (from 10 to 15 a) on the Loess Plateau, and Proteobacteria and Ascomycota were the dominant phyla for bacteria and fungi, respectively. However, during the later RP restoration period (from 15 to 40 a), soil bacterial diversity was significantly lower than that during the early RP restoration period, and the rate of increase in fungal diversity decreased. During the later RP restoration period, the relative abundances of Proteobacteria and Ascomycota were lower than those during the early RP restoration period, while the relative abundances of Actinobacteria, Acidobacteria and Basidiomycota were greater during this period. The variations in soil bacterial communities were mainly related to the changes in soil total N, nitrate N and SM, while soil fungal communities were mainly shaped by SOC and soil nitrate N. Bacterial communities were structured by the heterogeneous selection and stochastic process, while fungal communities were structured primarily by the stochastic process. The restoration of RP plantations induced an increase in the relative importance of the heterogeneous selection on bacterial communities. Overall, this study reveals the changes in microbial diversity, community compositions and assembly processes during the RP restoration period (40-a chronosequence) on the Loess Plateau and provides a new perspective for studying the effects of vegetation restoration on soil microbial communities in semi-arid regions.

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Appendix

Table S1 Geographical characteristics of *Robinia pseudoacacia* L. (RP) plantations with different stand ages

RP plantation	Location	Altitude (m)	ATH (m)	Slope gradient (°)	DBH (cm)	Trees density (numbers/hm^2)	Litter biomass (g/m^2)
Y10	109°28′E, 36°27′N	1276.4	6.92±0.24	20	6.40±0.42	1600	190.05±7.92
Y15	109°33′E, 36°27′N	1338.1	7.33±0.15	18	7.00±0.34	2800	418.39±11.58
Y25	109°29′E, 36°24′N	1351.6	9.87±1.21	16	15.30±1.27	1500	505.61±12.34
Y40	110°22′E, 38°47′N	1342.4	10.72±0.69	18	17.30±1.56	1300	556.88±17.60

Note: ATH, average tree height; DBH, diameter at breast height. Values of ATH and DBH represent the mean values of randomly selected 20 RP trees with DBH greater than 2.00 cm. The leaves of RP were collect using five collectors (1 m×1 m) and dried to constant weight at 65 ℃ to obtain the litter biomass. Y10, Y15, Y25 and Y40 indicate RP plantations with stand ages of 10, 15, 25 and 40 a, respectively. Mean±SD.

Table S2 Relationships between soil properties and microbial α diversity indices

Soil property	Bacterial α diversity		Fungal α diversity	
	Shannon	Chao	Shannon	Chao
pH	0.435	–0.016	–0.823**	–0.858**
Moisture	0.728**	0.556*	–0.233	–0.201
SOC	–0.754**	–0.269	0.762**	0.846**
TN	–0.760**	–0.255	0.765**	0.852**
NO_3^--N	0.516	–0.084	–0.255	–0.256
NH_4^+-N	–0.640**	–0.265	0.630**	0.698**
AP	–0.658**	–0.382	0.518*	0.545*
C:N	–0.421	–0.378	0.330	0.379

Note: SM, soil moisture; SOC, soil organic carbon; TN, total nitrogen; NO_3^--N, nitrate nitrogen; NH_4^+-N, ammonium nitrogen; AP, available phosphorous. **, P<0.01 level, *, P<0.05 level.

Table S3 Significance tests of the variations of fungal communities between Y10 and Y15 using the Adonis test

	df	Sums of square	Mean of square	*F*	*P*
Group factor	1	0.682	0.682	8.429	0.011
Residual	8	0.647	0.081		
Total	9	1.330			

Note: Significant level is P<0.05.

Table S4 Forward selection results of the redundancy analysis (RDA) for microbial communities and soil properties

Microbe	Soil property	Explanation (%)	Contribution (%)	*F*	*P*
Bacteria	TN$^{\#}$	21.5	39.2	4.9	0.002
	NO_3^--N$^{\#}$	10.0	18.2	2.5	0.002
	SM$^{\#}$	9.1	16.6	2.5	0.002
	pH	4.0	7.3	1.1	0.346
	SOC	3.8	6.9	1.0	0.408
	AP	3.3	6.1	0.9	0.654
	NH_4^+-N	3.1	5.7	0.8	0.718
Fungi	SOC$^{\#}$	28.9	43.2	7.3	0.002
	NO_3^--N$^{\#}$	17.6	18.8	3.7	0.002
	SM	5.1	7.2	1.8	0.059
	pH	4.4	6.6	1.5	0.078
	TN	5.8	8.6	2.1	0.014
	AP	2.7	4.0	1.0	0.462
	NH_4^+-N	2.4	3.6	0.9	0.632

Note: $^{\#}$, soil property was significantly affected by microbial communities. Significant level is P<0.05.

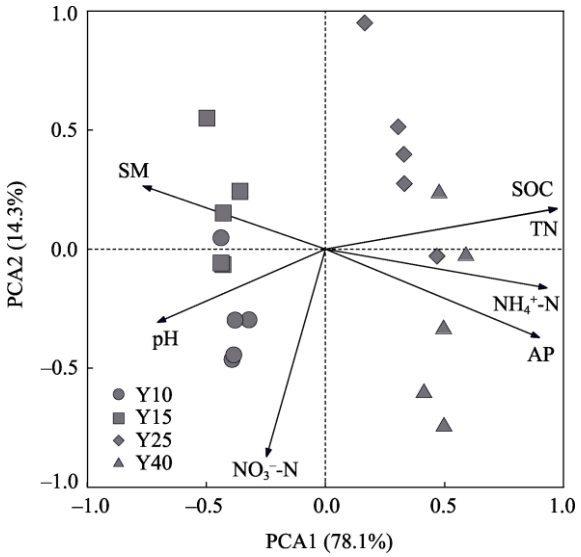

Fig. S1 Principal component analysis (PCA) of soil properties at four *Robinia pseudoacacia* (RP) plantations with different stand ages. PCA and visualization were performed using CANOCO v5.0. SM, soil moisture; SOC, soil organic carbon; TN, total nitrogen; NO_3^--N, nitrate nitrogen; NH_4^+-N, ammonium nitrogen; AP, available phosphorous.

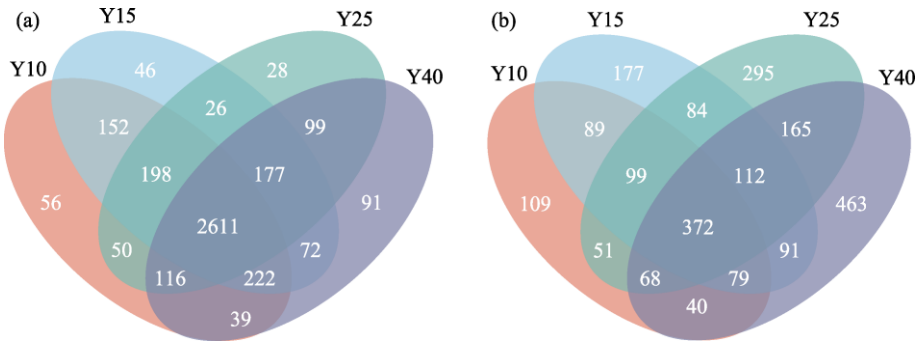

Fig. S2 Venn analyses of the operational taxonomic units (OTUs) of soil bacterial (a) and fungal (b) communities at four RP plantations with different stand ages. Venn analyses and visualization were performed using the "VennDiagram" package in R 3.4.1.

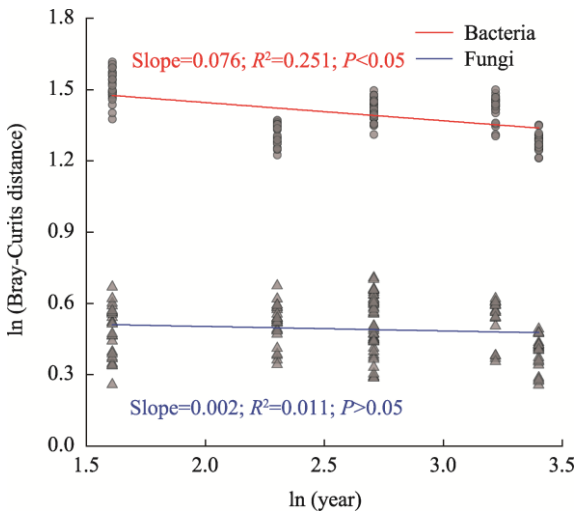

Fig. S3 Time-decay relationships of fungal and bacterial communities at RP plantations with 40 a of restoration. The slope means the temporal turnover rate of microbial communities across restoration time.